STANDARD OPERATING PROCEDURE

NHP GENOMICS CORE LABORATORY

Subject: Illumina TruSeq Stranded mRNA Sample Prep LS Protocol	**SOP Number:** 71 **Version:** 1
Effective Date: 01/01/2016	**Revised Date:** May 23, 2016

Illumina TruSeq Stranded mRNA Sample Prep Protocol

Note: This protocol is adapted from the Illumina TruSeq Stranded mRNA Sample Prep Protocol.

Rationale:

To describe the procedure for converting the mRNA in total RNA into a library of template molecules of known strand origin using the reagents provided in the Illumina® TruSeq® Stranded mRNA Sample Preparation Kits that are suitable for subsequent cluster generation and DNA sequencing.

Important points:

- Observe general principles for handling RNA to maximize RNA integrity during sampling. See the links below for more information:
  http://www1.qiagen.com/literature/handbooks/literature.aspx?id=1000276
  http://www.ambion.com/techlib/basics/rnasecontrol/index.html

- Use RNAse-free, sterile polypropylene tubes for storing the sample.
- **Sample Type:** 100ng – 4ug of high quality total RNA, RIN>8 required.
- If the total RNA was isolated from whole blood (Paxgene or Tempus) tubes, please follow the SOP#72.1 for globin depletion.
- Optional- If ERCC internal spike-in controls are used with this protocol follow SOP#69.1.

Equipment Requirements:

Materials	Company	Cat. No.
Agilent 2100 Bioanalyzer	Agilent	N/A
Nanodrop2000	Thermo	N/A
Agilent DNA 1000 Kit	Agilent	5067-1504, 5067-1505
Bioanalyzer Chip Vortex	IKA	N/A
Qubit 2.0 Fluorometer	Invitrogen	N/A
Thermal cycler	Bio Rad	N/A
Vortex	Fischer Scientific	N/A
Microcentrifuge	Eppendorf	N/A
Magnetic Stand	Ambion	AM10027
Timer		N/A
Mini Plate Spinner	Lab Net	N/A

Reagents Requirements:

Materials	Company	Cat. No.
TruSeq stranded mRNA kit	Illumina	RS-122-2101/02/03
Superscript II Reverse Transcriptase	Invitrogen	18064-014
Ethanol	Fisher Scientific	BP2818500
AmpureXP SPRI beads	Agencourt	A63880
RNase ZAP	Sigma-Aldrich	R2020
DNA Off	Takara	9036
Wet Ice	N/A	N/A

Thermocycler Programs: Check or add the below programs in the thermocycler before starting the actual procedures

S.No	Program	Steps
1	mRNA denaturation	• 65˚C for 5 minutes • 4˚C hold
2	mRNA elution 1	• 80˚C for 2 minutes • 25˚C hold
3	Elution 2-Frag-Prime	• 94˚C for 8 minutes • 4˚C hold
4	1st Strand	• Pre-heat lid option and set to 100˚C • 25˚C for 10 minutes • 42˚C for 15 minutes • 70˚C for 15 minutes • 4˚C hold
5	2nd Strand	• 16˚C for 1 hour
6	ATAIL70	• Pre-heat lid option and set to 100˚C • 37˚C for 30 minutes • 70˚C for 5 minutes • 4˚C hold
7	ALP	• 30˚C for 10 minutes

8	PCR	• Pre-heat lid option and set to 100˚C
		• 98˚C for 30 seconds
		• 15 cycles of
		  ○ 98˚C for 10 seconds
		  ○ 60˚C for 30 seconds
		  ○ 72˚C for 30 seconds
		• 72˚C for 5 minutes
		• 10˚C hold

A. Make RBP

1. Allow RNA Purification Beads (RPB), Bead Washing Buffer (BWB), Bead Binding Buffer (BBB), Elution Buffer (ELB) and Resuspension Buffer (RSB) to be at room temperature before you start the process.
2. Dilute the total RNA with nuclease-free ultra pure water to a final volume of 50µl in the new 96-well 0.3ml PCR plate labeled with the RBP barcode.
3. Vortex the RNA Purification Beads (RPB) to resuspend the oligo-dT beads.
4. Add 50µl of RNA Purification Beads (RPB) to each well and mix thoroughly using a vortex or a micropipette by gently pipetting entire volume up and down 6 times.
5. Seal the plate with Microseal 'B' adhesive seal.

B. RBP Incubation # 1

6. Place the sealed RBP plate on the thermal cycler and select the **mRNA Denaturation** program.
7. Remove the RBP plate from the thermal cycler when it reaches 4˚C.
8. Incubate the RBP plate at room temperature for 5 minutes to allow RNA to bind to the beads.

C. Wash RBP

9. Place the RBP plate on the magnetic stand and remove the adhesive seal.
10. Keep the plate on the magnetic stand at room temperature for 5 minutes to separate the polyA RNA bound beads from the solution.
11. Remove and discard the supernatant from each well.
12. Remove the RBP plate from the magnetic stand and place it on the 96 well plate holder.
13. Wash the beads with 200µl Bead Washing Buffer (BWB) in each well to remove unbound RNA.
14. Mix thoroughly by gently pipetting up and down for 6 times.
15. Place the RBP plate on the magnetic stand at room temperature for 5 minutes.
16. Remove and discard the supernatant from each well of RBP plate.
17. Remove the RBP plate from magnetic stand and add 50µl Elution Buffer in each well. Mix thoroughly.
18. Seal the plate with a Microseal 'B' adhesive seal.
19. Briefly centrifuge, vortex and centrifuge to mix and bring down the contents to the bottom of the well.

D. RBP Incubation # 2

20. Place the RBP plate on the thermocycler and select **mRNA Elution 1** program to elute mRNA from beads.
21. Remove the plate from the thermocycler when it reaches 25˚C and place it on the bench at room temperature.
22. Remove the seal carefully.

E. Making RNA Fragmentation Plate (RFP)

23. Add 50µl Bead Binding buffer to each well of the RBP plate.
24. Mix thoroughly with gentle pipetting.
25. Incubate the plate at room temperature for 5 minutes.
26. Place the RBP plate on the magnetic stand at room temperature for 5 minutes.
27. Remove and discard the supernatant from each well of RBP plate.
28. Remove the RBP plate from magnetic stand and add 200µl of Bead Washing Buffer (BWB) in each well and mix thoroughly with gentle pipetting.
29. Place the RBP plate on the magnetic stand at room temperature for 5 minutes.
30. Place the Fragment, Prime, Finish Mix on ice to thaw.
31. Remove and discard the supernatant from each well of RBP plate.
32. Remove the RBP plate from magnetic stand and add 19.5µl of Fragment, Prime, Finish Mix to each well.
33. Mix thoroughly by gentle pipetting for 6 times.
34. Seal the plate with a Microseal ‘B’ adhesive seal.
35. Briefly centrifuge, vortex and centrifuge to mix and bring down the contents to the bottom of the well.

F. Incubate RNA Fragmentation Plate (RFP)

36. Place the RBP plate on the thermal cycler and select **Elution 2 – Frag- Prime** program to elute, fragment and prime the RNA.
37. Remove the plate from the thermal cycler when it reaches 4˚C and centrifuge briefly.
38. Proceed immediately to the next step.

G. Make cDNA Plate (CDP)

39. Place the RBP plate on the magnetic stand at room temperature for 5 minutes.
40. Place the First Strand Synthesis Act D Mix and SuperScript II mix contents on ice to thaw.
41. Remove the adhesive seal keeping the plate on magnetic stand.
42. Transfer 17µl of the supernatant from each well of RBP plate to the corresponding well in the new 96-well PCR plate labeled as CDP.

43. Prepare First Strand Synthesis Act D Mix and SuperScript II mix by adding 50µl SuperScript II to the First Strand Synthesis Act D Mix tube (Ratio: 1µl SuperScript II for each 9µl First Strand Synthesis Act D Mix).
44. Add 8µl of the First Strand Synthesis Act D and SuperScript II Mix to each well.
45. Seal the plate with a Microseal 'B' adhesive seal.
46. Briefly centrifuge, vortex and centrifuge to mix and bring down the contents to the bottom of the well.

H. CDP Incubation # 1

47. Place the sealed CDP plate on the thermal cycler and select **1st Strand** program.
48. Bring AMPure beads from 4˚C and let stand at room temperature for at least 30 minutes.
49. Take out the Second Strand Master Mix and End Repair Control from storage to thaw.
50. Remove the CDP plate from thermal cycler when the temperature is 4˚C and proceed immediately to the next step.

I. Add SSM

51. Centrifuge thawed End Repair Control tube to 600 xg for 5 seconds and dilute the Control to 1/50 in Resuspension Buffer (2µl End Repair Control + 98µl Resuspension Buffer).
52. Carefully remove the adhesive seal.
53. Add 5µl of diluted End Repair Control to each well of CDP plate. For Negative Control well, add 5µl of Resuspension Buffer instead of End Repair control.
54. Add 20µl of Second Strand Master Mix to each well of CDP plate.
55. Mix thoroughly by gentle pipetting for 6 times.
56. Seal the plate with a Microseal 'B' adhesive seal.
57. Brief centrifuge, vortex and centrifuge to mix and bring down the contents to the bottom of the well.

J. CDP Incubation # 2

58. Place the plate in the pre-heated thermo cycler for 1 hour at 16˚C. *Do not place the plate in the thermo cycler until the temperature has reached 16˚C*.
59. Remove the CDP plate from the thermo cycler.
60. Remove the adhesive seal from the CDP plate.
61. Let the CDP plate come to the room temperature.

K. Purify CDP

62. Vortex the AMPure XP beads until they are well dispersed.
63. Add 90µl of AMPure XP beads to each well of the CDP plate.

64. Gently pipette up and down for 10 times to mix thoroughly.
65. Incubate the plate at room temperature for 15 minutes.
66. Place the CDP plate on the magnetic stand for 5 minutes.
67. Remove and discard 135µl of supernatant from each well.
68. With the CDP plate on the magnetic stand, add 200µl of freshly prepared 80% ethanol to each well without disturbing the beads (Ethanol wash # 1).
69. Incubate the plate at room temperature for 30 seconds.
70. Remove and discard all the supernatant from each well.
71. With the CDP plate on the magnetic stand, add 200µl of freshly prepared 80% ethanol to each well without disturbing the beads (Ethanol wash # 2).
72. Incubate the plate at room temperature for 30 seconds.
73. Remove and discard all the supernatant from each well.
74. Make sure that all the Ethanol is removed from the wells of the CDP plate.
75. Keep the CDP plate at room temperature for 15 minutes to dry.
76. Remove the plate from the magnetic stand.
77. Add 52.5µl Resuspension buffer to each well of the CDP plate.
78. Gently pipette the entire volume up and down 10 times to mix thoroughly.
79. Incubate the CDP plate at room temperature for 2 minutes.
80. Place the CDP plate on the magnetic stand at room temperature for 5 minutes.
81. Transfer 15µl of the supernatant from the CDP plate to the new 0.3 ml ALP plate labeled as IMP.
82. SAFE STOPPING POINT. Can store sealed plate at -25˚C to -15˚C for up to 7 days.

L. Preparation for Adenylate 3'Ends

83. Thaw
    - ALP Plate and briefly centrifuge for 5 seconds
    - A-Tailing Mix
    - A-Tailing Control
84. Preheat the thermal cycler lid to 30˚C.

M. Add ATL

85. Centrifuge thawed A-Tailing Control tube to 600 xg for 5 seconds and dilute the A-Tailing Control to 1/100 in Resuspension Buffer (1µl A-Tailing Control + 99µl Resuspension Buffer).
86. Keep the diluted Control tube on wet ice.
87. Add 2.5µl of diluted A-Tailing Control to each well of ALP plate. For Negative Control well, add 2.5µl of the Resuspension Buffer instead of the A-Tailing Control.
88. Discard the diluted A-Tailing Control after use.
89. Add 12.5µl of A-Tailing Mix to each to all the wells of ALP plate.
90. Mix thoroughly by gentle pipetting for 10 times.

91. Seal the ALP plate with a Microseal ‘B’ adhesive seal.

N. Incubate #1 ALP

92. Incubate the ALP plate on the pre-heated thermal cycler, with the lid closed and choose **ATAIL70**.
93. Remove the ALP plate from the thermal cycler when it reaches 4˚C and proceed immediately to *Ligate Adapters* steps.

O. Preparation for the Ligate Adapters

94. Thaw
    - RNA Adapter Index tubes or RAP plate if using the HT version of the kit
    - Ligation Control
    - Stop Ligation Buffer
95. Briefly centrifuge the above.
96. Bring AMPure beads from 4˚C and let stand at room temperature for at least 30 minutes.
97. Preheat the thermocycler lid to 30˚C.
98. Ligation Mix is prepared in glycerol. It is not frozen. Remove the tube just before adding it to the wells. Return it to freezer immediately after use.

P. Add LIG

99. Dilute the Ligation Control to 1/100 in Resuspension Buffer (1µl Ligation Control + 99µl Resuspension Buffer).
100. Keep the diluted Control tube on wet ice.
101. Add 2.5µl of diluted Ligation Control to each well of ALP plate.
102. Discard the diluted Ligation Control after use.
103. Add 2.5µl of Ligation Mix to each well of ALP plate.
104. Add 2.5µl of thawed RNA Adapter Index to respective well of ALP plate (**Note:** Each sample gets a unique index if being pooled and run in the same lane). If using the HT kit thaw, vortex and centrifuge the RAP plate briefly. Add 2.5 ul of pre-mixed reverse and forward primer from the RAP plate to the respective well of the ALP plate.
105. Mix thoroughly by gentle pipetting for 10 times.
106. Seal the ALP plate with a Microseal ‘B’ adhesive seal.

Q. Incubate #2 ALP

107. Incubate the ALP plate on the pre-heated thermal cycler, with the lid closed and choose **ALP** Program.
108. Remove the ALP plate from the thermal cycler.

R. Add STL

109. Remove the Adhesive seal.
110. Add 5µl of Stop Ligation Buffer to each well of ALP plate to stop the Ligation activity.
111. Mix thoroughly by gentle pipetting for 10 times.

S. Clean Up ALP

112. Vortex the AMPure XP beads until they are well dispersed.
113. Add 42µl of AMPure XP beads to each well of the ALP plate.
114. Gently pipette up and down for 10 times to mix thoroughly.
115. Incubate the plate at room temperature for 15 minutes.
116. Place the ALP plate on the magnetic stand at room temperature for 5 minutes until the liquid is clear.
117. Remove and discard 79.5µl of supernatant from each well.
118. With the ALP plate on the magnetic stand, add 200µl of freshly prepared 80% ethanol to each well without disturbing the beads (Ethanol wash # 1).
119. Incubate the plate at room temperature for 30 seconds.
120. Remove and discard all the supernatant from each well.
121. With the ALP plate on the magnetic stand, add 200µl of freshly prepared 80% ethanol to each well without disturbing the beads (Ethanol wash # 2).
122. Incubate the plate at room temperature for 30 seconds.
123. Remove and discard all the supernatant from each well.
124. Make sure that all the Ethanol is removed from the wells of the ALP plate.
125. Keep the ALP plate at room temperature for 15 minutes to dry.
126. Remove the plate from the magnetic stand.
127. Add 52.5µl Resuspension buffer to each well of the ALP plate.
128. Gently pipette the entire volume up and down 10 times to mix thoroughly until the beads are fully resuspended.
129. Incubate the ALP plate at room temperature for 2 minutes.
130. Place the ALP plate on the magnetic stand at room temperature for 5 minutes until the liquid is clear.
131. Transfer 50µl of the supernatant from each well of the ALP plate to the new 0.3 ml PCR plate labeled with CAP barcode.

T. Clean Up CAP

132. Vortex the AMPure XP beads until they are well dispersed.
133. Add 50µl of AMPure XP beads to each well of the CAP plate for second cleanup.
134. Gently pipette up and down for 10 times to mix thoroughly.
135. Incubate the plate at room temperature for 15 minutes.

136. Place the CAP plate on the magnetic stand at room temperature for 5 minutes until the liquid is clear.
137. Remove and discard 95µl of supernatant from each well.
138. With the CAP plate on the magnetic stand, add 200µl of freshly prepared 80% ethanol to each well without disturbing the beads (Ethanol wash # 1).
139. Incubate the plate at room temperature for 30 seconds.
140. Remove and discard all the supernatant from each well.
141. With the CAP plate on the magnetic stand, add 200µl of freshly prepared 80% ethanol to each well without disturbing the beads (Ethanol wash # 2).
142. Incubate the plate at room temperature for 30 seconds.
143. Remove and discard all the supernatant from each well.
144. Make sure that all the Ethanol is removed from the wells of the CAP plate.
145. Keep the CAP plate at room temperature for 15 minutes to dry.
146. Remove the plate from the magnetic stand.
147. Add 22.5µl Resuspension buffer to each well of the CAP plate.
148. Gently pipette the entire volume up and down 10 times to mix thoroughly until the beads are fully resuspended.
149. Incubate the CAP plate at room temperature for 2 minutes.
150. Place the CAP plate on the magnetic stand at room temperature for 5 minutes until the liquid is clear.
151. Transfer 20µl of the supernatant from each well of the CAP plate to the corresponding well of the new 0.3 ml PCR plate labeled with PCR barcode.
152. SAFE STOPPING POINT. Can store sealed plate at -25˚C to -15˚C for up to 7 days.

U. Enrich DNA Fragments

153. Thaw
    - PCR Master Mix
    - PCR Primer Cocktail
    - PCR Plate
154. Briefly centrifuge the above.
155. Bring AMPure beads from 4˚C and let stand at room temperature for at least 30 minutes.

V. Make PCR

156. Add 5µl of PCR Primer Cocktail to each well of PCR plate.
157. Add 25µl of PCR Master Mix to each well of PCR plate.
158. Gently pipette the entire volume up and down 10 times to mix thoroughly.
159. Seal the PCR plate with a Microseal ‘B’ adhesive seal.

W. AMP PCR

160. Place the sealed plate on the pre-programmed thermocycler and choose the **PCR** option to amplify the plate.

X. Clean Up PCR

161. Remove adhesive seal from the PCR plate.
162. Vortex the AMPure XP beads until they are well dispersed.
163. Add 50µl of AMPure XP beads to each well of the PCR plate containing 50µl of amplified libraries.
164. Gently pipette up and down for 10 times to mix thoroughly.
165. Incubate the plate at room temperature for 15 minutes.
166. Place the PCR plate on the magnetic stand at room temperature for 5 minutes until the liquid is clear.
167. Remove and discard 95µl of supernatant from each well.
168. With the PCR plate on the magnetic stand, add 200µl of freshly prepared 80% ethanol to each well without disturbing the beads (Ethanol wash # 1).
169. Incubate the plate at room temperature for 30 seconds.
170. Remove and discard all the supernatant from each well.
171. With the PCR plate on the magnetic stand, add 200µl of freshly prepared 80% ethanol to each well without disturbing the beads (Ethanol wash # 2).
172. Incubate the plate at room temperature for 30 seconds.
173. Remove and discard all the supernatant from each well.
174. Make sure that all the Ethanol is removed from the wells of the PCR plate.
175. Allow the PCR plate to dry at room temperature for 15 minutes keeping it on the magnetic stand.
176. Remove the plate from the magnetic stand.
177. Add 32.5µl Resuspension buffer to each well of the PCR plate.
178. Gently pipette the entire volume up and down 10 times to mix thoroughly.
179. Incubate the PCR plate at room temperature for 2 minutes.
180. Place the PCR plate on the magnetic stand at room temperature for 5 minutes until the liquid is clear.
181. Transfer 10µl of the supernatant from each well of the PCR plate to the new 0.3 ml PCR plate with DCT barcode on it.
182. Transfer 16µl of the supernatant from each well of the PCR plate to the new 0.3 ml PCR plate with TSP1 barcode on it.
183. Transfer 4µl of the supernatant from each well of the PCR plate to the new 0.3 ml PCR plate to validate the libraries for quantification and quality check.
184. SAFE STOPPING POINT. Can store sealed plate at -25˚C to -15˚C for up to 7 days.

Y. Validate Library

185. Quantification is done using Qubit.
186. Quality Control by sizing and analysis of DNA fragments is performed using Bioanalyzer using a DNA specific chip.

Quantification using Qubit 2.0 Fluorometer

Requirements

- Qubit dsDNA HS reagent
- Qubit dsDNA HS buffer
- Qubit dsDNA HS Standard #1
- Qubit dsDNA HS Standard #2
- Qubit Assay tubes
- Qubit 2.0 Fluorometer

187. Prepare Qubit working solution by adding Qubit dsDNA HS reagent to Qubit dsDNA HS Buffer in the ratio of 1:200.
188. For standards, add 10µl of standard solution to 190µl of dsDNA HS Qubit working solution.
189. For samples, add 2µl of sample to 198µl of dsDNA HS Qubit working solution.
190. The total volume in each assay tube must be 200µl.
191. Vortex the tubes for 2-3 seconds.
192. Incubate the tubes for 2 minutes at room temperature.
193. Read the concentrations of the standards first on the Qubit 2.0 Fluorometer.
194. Then read the concentrations of the samples. Adjust the sample volume as used.
195. Check the required concentration units and save the data.

Quality Check using 2100 Agilent Bioanalyzer

Requirements

- Chip Priming Station
- Agilent DNA 1000 Chip
- Gel-Dye Mix
- Marker
- DNA 1000 Ladder

Preparation of Gel-Dye Mix

196. Allow DNA 1000 dye concentrate and DNA 1000 gel matrix to equilibrate to the room temperature for 30 minutes.
197. Add 25µl of DNA 1000 dye concentrate to DNA 1000 gel matrix vial.
198. Vortex well and spin down.
199. Transfer to spin filter.
200. Centrifuge at 2240 g ± 20 % for 10 min.

Loading the Gel-Dye Mix

201. Allow the gel-dye matrix to equilibrate to the room temperature for 30 minutes before use.
202. Put the DNA 1000 Chip on the Priming Station.
203. Pipette 9µl of gel-dye mix in the well marked G.
204. Make sure that the plunger is positioned at 1 ml and then close the chip priming station.
205. Press plunger until it is held by the clip.
206. Wait for exactly 60s and then release the clip.
207. Wait for 5s and slowly pull the plunger back to 1ml position.
208. Open the chip priming station and pipette 9µl of gel-dye mix in the wells marked **G**.

Loading the Marker

209. Pipette 5µl of marker in all sample and ladder wells.
210. Do not leave wells empty.

Loading the Ladder and Samples

211. Pipette 1µl of DNA 1000 Ladder in the well marked .
212. In each of the 12 sample wells pipette 1µl of sample.
213. Put the chip horizontally in the adapter and vortex for 1 min at 2200 rpm at room temperature.
214. Run the chip in the Agilent 2100 Bioanalyzer instrument within 5 min.

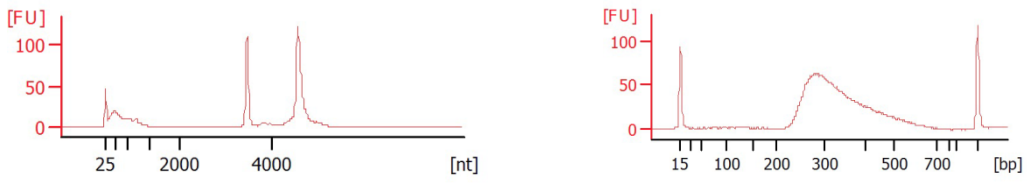

Figure 1. Representative Bioanalyzer traces for RNA and sequencing libraries.

Z. Normalize and Pool Libraries

215. Normalize the concentration of sample library in each well of DCT plate to 10nM using EB-Tween (Tris-HCl 10mM, pH 8.5 with 0.1% Tween 20).
216. Gently pipette the sample library up and down 10 times to mix thoroughly.

Make PDP

217. Determine the number of samples to be combined in each pool.

218. Transfer 10µl of each normalized sample library to be pooled from the DCT plate to a new 0.3 ml PCR plate labeled with the PDP barcode.
219. Pipette the entire volume up and down 10 times to mix.
220. Do one of the following:
    - Proceed to cBOT or
    - Seal the PDP plate with a Microseal ‘B’ adhesive seal and store at -15˚C to -25˚C.